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Author(s): Suling Zhao, Sarah Maxwell, Antonio Jimenez-Beristain, Joaquim Vives, Eva Kuehner, Jiexin Zhao, Carmel O'Brien, Carmen de Felipe, Elena Semina and Meng Li
Journal: European Journal of Neuroscience
ISSN: 0264-1615
Year: March 2004 **Volume:** 19 **Issue:** 5 **Page:** 1133 - 1400
DOI: 10.1111/j.1460-9568.2004.03206.x

Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons

Suling Zhao[1#], Sarah Maxwell,[1#] Antonio Jimenez-Beristain[2#], Joaquim Vives[1], Eva Kuehner[1], Jiexin Zhao[1], Carmel O'Brian[3], Carmen de Felipe[2], Elena Semina[4] and Meng Li[1]*

[1]Institute for Stem Cell Research, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh, United Kingdom, EH9 3JQ, UK

[2]Instituto de Neurociencias, Universidad Miguel Hernandez-Consejo Superior de Investigaciones Cientificas, Alicante, Spain.

[3]Stem Cell Sciences ltd, Melbourne 3066, Victoria, Australia.

[4]Department of Paediatrics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

[#] These authors contributed equally to this work

*Author for correspondence: Meng.Li@ed.ac.uk

Tel. +44 131 650 5890

Fax. +44 131 650 7773

Abstract

We have generated embryonic stem cells and transgenic mice with green fluorescent protein (GFP) inserted into the *Pitx3* locus via homologous recombination. In the central nervous system, *Pitx3* directed GFP was visualised in dopaminergic (DA) neurons in the substantia nigra and ventral tegmental area. Live primary DA neurons can be isolated by fluorescence-activated cell sorting from these transgenic mouse embryos. In culture, *Pitx3*-GFP is co-expressed in a proportion of ES-derived DA neurons. Furthermore, stem cell-derived *Pitx3*-GFP expressing DA neurons responded to neurotrophic factors and were sensitive to DA specific neurotoxin N-4-methyl-1, 2, 3, 6-tetrahydropyridine. We anticipate that the *Pitx3*-GFP ES cells could be used as a powerful model system for functional identification of molecules governing mDA neuron differentiation and for pre-clinical research including pharmaceutical drug screen and transplantation. The *Pitx3* knock-in mice, on the other hand, could be used for purifying primary neurons for molecular studies associated with the midbrain-specific DA phenotype at a level not previously feasible. These mice would also provide a useful tool to study DA fate determination from embryo- or adult-derived neural stem cells.

Key words: Dopaminergic neuron, green fluorescent protein (GFP), homologous recombination, homeobox transcription factor, Pitx3, stem cell.

Introduction

Parkinson's disease (PD) is one of the most debilitating neurological disorders that currently lack effective treatments. It is associated with the loss of midbrain dopaminergic (mDA) neurons. Studies in recent years have led to the recognition of factors establishing and patterning the midbrain region (Sonic hedgehog, FGF8, *Wnt1*, *Engrailed-1&2*) as well as transcription controls of DA transmitter phenotype (*Nurr1*) and survival of developing mDA neurons (*Nurr1* and *Lmx1b*) (McMahon et al., 1992; Saucedo-Cardenas et al., 1998; Simon et al., 2001; Smidt et al., 2000; Ye et al., 1998; Zetterstrom et al., 1997). However, signalling and transcriptional events leading neural progenitors to the generation of postmitotic mDA neurons and subsequent maintenance of these neurons in the adult brain are still poorly understood.

ES cells are non-transformed, pluripotent cell lines derived directly from the inner cell mass of the pre-implantation embryo (Evans and Kaufman, 1981). These cells have the inherent capacity to participate fully in embryonic development when reintroduced into blastocyst. *In vitro* ES cells can give rise to derivatives of all three primary germ layers in a way that is suggested to recapitulate events of embryogenesis (Doetschman et al., 1985). Therefore, ES cells provide an important cellular system for analysis of neural development allowing the processes of fate determination and differentiation to be dissected in detail, which otherwise are difficult to study in experimental animal systems. Since ES cells are easily accessible for genetic modification without compromising pluripotency, they can be used to test certain transgene expression during differentiation

(Aubert et al., 2002; Kim et al., 2003). In addition, ES cells can potentially be used for drug discovery and to provide an unlimited source for cell based therapy for treating Parkinson's disease (Barberi et al., 2003; Kawasaki et al., 2000; Lee et al., 2000).

Pitx3 is a *paired*-like homeobox transcription factor that, within the central nervous system, is expressed exclusively in the substantia nigra (SN) and the ventral tegmental area (VTA) that together form the mDA system (Smidt et al., 1997). Midbrain expression of *Pitx3* transcripts is first seen at E11 and is maintained throughout life in both rodents and humans (Smidt et al., 1997). Unlike *Nurr 1* or *Lmx1b*, which display a broader expression pattern in the central nervous system, Pitx3 expression is confined to mDA neurons (Smidt et al., 1997; Van Den Munckhof et al., 2003). Recent studies of *aphakia* mutant mice, in which *Pitx3* expression is strongly hypomorphic due to deletions of the presumed *Pitx3* promoter regions, showed that mDA neurons are lost in these mice (Hwang et al., 2003; Nunes et al., 2003; Rieger et al., 2001; Semina et al., 2000b; Van Den Munckhof et al., 2003). These studies suggest that *Pitx3* is not only a specific marker for mDA neurons; it is also functionally required by mDA neurons for their development and maintenance. Therefore, a model system where *Pitx3* directed expression could be monitored would be important to define factors that regulate mDA phenotype.

In vitro differentiation of ES cells in combination with an engineered lineage marker can provide as a useful tool to evaluate molecules that regulate cell fate and/or differentiation (Aubert et al., 2002; Li et al., 1998; Ying et al., 2003). Here we report the generation of

ES cells and a transgenic mouse line that express *Pitx3* regulated GFP reporter in mDA system.

Materials and methods

Pitx3 targeting vector

A *Pitx3* genomic clone was isolated from a mouse BAC library (Research Genetics). To integrate a *Pitx3* directed eGFP reporter as well as to generate a *Pitx3*-deficient locus, DNA sequence including part of intron 1, coding exons 2, 3 and half of exon 4 was replaced with an engrailed 2 splice acceptor-eGFP-ires-pac-pA cassette (SA-eGFP-ires-pac, *pac* is puromycin resistant gene) (Mountford et al., 1994). For selection of ES cell transfectants following electroporation, a floxed PGK promoter driven *hygromycin-thymidine kinase* (PGK-hygro-tk) fusion gene was placed downstream of the eGFP reporter cassette. The targeting vector consisted of a 1.8 kb 5' homology and a 6 kb 3' homology (Fig 1A).

ES cell culture and gene targeting

ES cells were maintained in GMEM supplemented with 2-mercaptoethanol, non-essential amino acids, sodium bicarbonate, 10% fetal calf serum (FCS) and 100 units/ml LIF, on gelatinised tissue culture flasks (Smith, 1991). For electroporation 100 μg of linearized targeting vector was electroporated into E14TG2a ES cells. Genomic DNA from 284 hygromycin resistant clones was digested with Hind3 and Southern blotted using a 5' external probe. Four homologous recombinants were obtained. One of the recombinant clones was then electroporated with a Cre expressing vector followed by selection with

gancyclovir. Following confirmation of deletion of the PGK-hygro-tk cassette, ES cells from three independent sub-clones were injected into blastocysts in two separate experiments. Each blastocyst received 10-20 ES cells and a total of 42 blastocysts were injected. 20 pups were born and 12 of which had various degree of ES cell contribution as judged by coat colour. Three best chimeric males were mated with C57BL/6 females and germline transmission of the targeted *Pitx3* allele was obtained, as confirmed by Southern blotting of tail tip DNA from the resultant progeny.

In vitro differentiation

In vitro differentiation of ES cells on PA6 stromal cells were carried out as previously described (Kawasaki et al., 2000). Briefly, PTG2 ES cells were cultured on a layer of PA6 stromal cells for 7 days in GMEM supplemented with knock-out serum replacement at 70 cells per cm^2. From day 7 the above medium was replaced with DMEM/F12 supplemented with N2 (Gibco) and B27 (Gibco) with or without added neruotrophic factors or Sonic Hedgehog (Shh) and FGF8. Trophic factors were maintained for 7 days (replenished every 2 days) whilst Shh and FGF8 were added for 4 days followed by GDNF till the end of the culture. For MPTP (Sigma) experiment, cultures were exposed to 100mM of MPTP for 48 hrs from day 12. Cultures were terminated at day 14 and processed for immunostaining. The concentration of added factors are: Shh 200ng/ml (R&D), GDNF, 5 ng/ml (R&D Systems), PDGF-BB, 20 ng/ml, NT3, 10 ng/ml, IGF1, 20 ng/ml, TGFα, 20 ng/ml, BDNF, 10 ng/ml EGF, 10 ng/ml FGF4, 20 ng/ml, FGF8 20ng/ml (all from Prepotech).

Immunostaining of cultured cells and brain sections

Cultures were washed once in Tris-buffered saline (TBS) then fixed in 4% paraformaldehyde for 10 minutes. Brains were fixed by immersion in 4% paraformaldehyde overnight, cryoprotected with 30% sucrose, sectioned at 30 μm on a cryostate and collected in PBS. Fixed cells and floating sections were then blocked with 3% normal serum, 1%BSA and 0.2% Triton X-100 in TBS and were incubated with primary antibodies (TH, 1:1000, rabbit, Pel Freeze; βTubulin3, 1:400, mouse IgG, Sigma; DAT, 1:500, rabbit, Chemicon; Sox1, rabbit , 1:500 CeMine, GFP, chicken, 1:2500, Chemicon) in blocking solution at 4°C overnight. Cells/sections were washed three times for 20 minutes each in TBS + 1% BSA, then incubated with fluorescence-labelled secondary antibodies for 1 h at room temperature (overnight at 4°C for sections). After washing three times with TBS, cell/sections were mounted in Vectashield (Vector) and analyzed using a Zeiss Axiophot microscope or Leica confocal microscope.

Mesencephalic cell preparation and FACS sorting

Brains were dissected from embryos and the midbrain region was excised and placed in GMEM. Tissues were chopped into small pieces and were incubated with 0.25 mg/ml collagenase type IA (Sigma) for 60 min at 37°C in GMEM. Collagenase-digested midbrains were then triturated in a drawn-out Pasteur pipette to dissociate the cells in medium containing 10% FCS. After passing through a 70 μm cell strainer, cells were collected by centrifugation and resuspended in ice-cold PBS. FACS sorting of GFP^+ cells was performed on a FACS MoFlo cell sorter (DAKO Cytomation). Dead cells were excluded by gating on forward and side scatter. Forebrain cell preparations (which do not contain GFP^+ cells) were used as negative control.

Results

Targeting of eGFP into the *Pitx3* locus

To generate *Pitx3*-linked GFP ES cells and mice, a targeting vector that contains a splice acceptor-eGFP-ires-pac-pA cassette, a loxP flanked PGK-*hygro-tk* cassette and a total of 8 kb *Pitx3* homologous arm was used to replace exons 2, 3 and half of exon 4 of the *Pitx3* gene (Fig 1). Four targeted ES clones were obtained from 284 clones of ES cell transfectants. One of the targeted clones was then electroporated with a Cre expressing vector for excision of the loxP flanked PGK-*hygro-tk* cassette (Aubert et al., 2003). Mice were generated from three of the PGK-*hygro-tk* deleted ES clones. Similar to heterozygous *aphakia* mice, heterozygous (*Pitx3*$^{GFP/+}$) mice, which were used in this study, were grossly normal and fertile.

Pitx3-directed GFP expression mirror that of *Pitx3* transcripts during embryonic development

The expression of *Pitx3* RNA has been reported in ventral mesencephalon, lens, tongue, mesenchyme around the sternum, vertebrae and head muscles (Semina et al., 1998; Semina et al., 2000b; Smidt et al., 1997). To investigate the fidelity of *Pitx3*-GFP reporter regulation, we examined the expression of *Pitx3*-GFP in *Pitx3*$^{GFP/+}$ mouse embryos. The first sign of *Pitx3*-GFP expression was found in the lens and somites of day 10.5 post coitum (E10.5) mouse embryos (Fig. 2A, B and D). Lens expression of *Pitx3*-GFP was maintained throughout embryonic development and persisted in adulthood (Fig 2D and data not shown). In agreement with *Pitx3* RNA expression profile, within the central

nervous system *Pitx3*-GFP is only seen in the midbrain region (Smidt et al., 1997). GFP signal was observed in a small area at the ventral most of E12 mesencephalon (Fig 2C). From E12.5 to E13.5, there was a significant increase in the size of GFP expressing domain in the ventral midbrain (Fig 2E). In E14 brain, both the cell bodies reside in the ventral midbrain and their axonal projections descending to the striatum and the basal forebrain were clearly visible (Fig 2F). In addition to lens and the midbrain, *Pitx3*-GFP expression was also found in the head mesenchyme, muscles in the cranial facial region and tongue (Fig 2C, 2G). All these *Pitx3*-GFP expression regions matched sites where expression of *Pitx3* transcripts was previously reported (Semina et al., 1998; Semina et al., 2000b; Smidt et al., 1997).

***Pitx3*-GFP is a specific marker for adult mDA neurons**

To determine in detail *Pitx3*-GFP expression in mDA neurons, we examined co-localisation of *Pitx3*-GFP with TH in SN and VTA on brain sections of the adult *Pitx3*$^{GFP/+}$ mice (Fig 3). In these experiments, an anti-GFP antibody was employed to intensify signals in GFP expressing cells. TH staining was found in SN and VTA of the midbrain, cells in paraventricular thalamic nucleus in diencephalon and in locus ceruleus in the brain stem (Fig 3B, E, H, K and data not shown). Immunostaining the same series of sections with the GFP antibody labelled only the TH positive cells in SN and VTA (Fig 3A, D, G, J). These data demonstrate that *Pitx3*-GFP expression recapitulate that of *Pitx3* transcripts in mDA neurons (Smidt et al., 1997). It has been reported recently that Pitx3 protein is differentially localised in DA neurons of the ventral SN and in only around 50% DA neurons of the VTA (Van Den Munckhof et al., 2003). However, this

did not seem to be the case for *Pitx3*-GFP since the vast majority of TH labelled cells were GFP positive and vice versa (Fig 3). As shown in table 1, 98.35±2.3% and 94.6±8.5% of TH^+ cells were *Pitx3*-GFP^+ in SN and VTA, respectively (Table 1). On the other hand, 99.36±0.9% (SN) and 94.9±6.4% (VTA) *Pitx3*-GFP^+ cells were TH^+. Those few single TH^+ or *Pitx3*-GFP^+ cells appeared to be randomly scattered within the SN or VTA (Fig 3A, D &E).

Expression of *Pitx3*-GFP in developing ventral mesencephalon

Prominent midbrain expression of *Pitx3* RNA was observed from E11 in the mouse embryo (Semina et al., 2000b; Smidt et al., 1997), which appears to be 0.5-1 day earlier than that of the TH (Pickel et al., 1975). In order to compare directly the kinetics of Pitx3-GFP and TH expression, we performed double antibody staining for GFP and TH on coronal sections of *Pitx3*$^{GFP/+}$ heads during the period of late mDA neuron differentiation. We began from E12 since it is the first day when *Pitx3*-GFP could be visualised. At this stage, only 56.3±6.7% of *Pitx3*-GFP^+ cells co-expressed TH whilst the rest were TH^- (Fig 4 A-C. G). By E14, most of *Pitx3*-GFP^+ cells (90.1±3.8%) were TH^+ (Fig 4D-F. G). The significant rise in the number of *Pitx3*-GFP^+ cells that express TH in developing ventral midbrain suggests that *Pitx3*-GFP expression precedes that of TH, at least in some mDA neurons. To further evaluate the developmental stage of *Pitx3*-GFP^+ cells in the E12 midbrain, we performed double antibody staining for GFP and Sox1, a marker that is expressed in mitotic neural progenitors (Pevny et al., 1998). We found that Sox1 and GFP expression is mutually exclusive in midbrain neuroepithelum, suggesting that *Pitx3*-GFP^+ TH^- cells were likely post-mitotic, late mDA precursors.

Direct isolation of mDA progenitor neurons by fluorescence activated cell sorting (FACS)

Flow cytometry is extensively used for the isolation of discreet populations of cells from complex pools. Non-invasive visualisation of *Pitx3*–GFP opens the possibility for direct isolation of primary mDA neurons from mice. We have therefore assessed this approach in *Pitx3*$^{GFP/+}$ embryos. The midbrain regions of E12-E16 embryos were dissected and single cell suspension prepared (Fig 5 and Table 2). Flow cytometry analysis revealed that these mesencephalic cell preparations contained *Pitx3+* cells in a range of 0.5-4%. In average, a total of 537 ± 271 GFP expressing neurons could be recovered per E12 embryonic midbrain from FACS sorting. A greater number of DA neurons could be obtained from older embryos, however (Table 2).

***Pitx3*-GFP is expressed in a sub-population of ES cell-derived DA neurons**

The expression analysis of *Pitx3-GFP* in developing embryos and the adult brain suggest that *Pitx3*-GFP provides a specific reporter to track mDA neuron differentiation from ES cells. To further evaluate this model system, PTG2 ES cells were differentiated by co-culture with PA6 stromal cells, a differentiation paradigm previously reported (Kawasaki et al., 2000). Under this culture condition, GFP positive cells with neuronal morphology can be readily detected at day 13-14 of differentiation although rare GFP expressing cells could be observed as early as day 7. GFP expression was primarily localised in cell bodies whereas fluorescence in neuronal processes were not as prominent (Fig 6A). The majority of GFP expressing cells were generated in close vicinity as clusters where most

of the cells express pan neuronal marker βTubulin 3 (97.7 %) and DA neuron marker TH (91.3%, Fig 6B and table 3). Patterning molecules such as Shh and FGF8 can promote the production of *Pitx3*-GFP$^+$ and/or TH$^+$ cells, as reported previously (Table 3)(Barberi et al., 2003; Lee et al., 2000). Furthermore, an average of 34.5% of *Pitx3*$^+$ cells also expressed dopamine transporters (DAT, Table 3). However, many TH and DAT expressing clusters contained few or no *Pitx3-GFP*$^+$ neurons (Fig 6C and data not shown). Overall, *Pitx3*-GFP$^+$ TH$^+$ and *Pitx3*-GFP$^+$ DAT$^+$ neurons accounted for 13.6% and 15.6% of total TH$^+$ or DAT$^+$ neurons, respectively. These data suggest that not all ES cell derived dopaminergic neurons exhibit mesencephalic identity, at least using the PA6 stromal cell system.

Neurotrophic factors promote the production of ES-derived mDA neurons and protect these cells from MPTP-induced cell death

Many neurotrophic factors have been shown to support the survival and differentiation of DA neurons from developing mesencephalon (Beck, 1994). To ask whether ES-derived mDA neurons behave similarly to their embryonic counter part, we tested several neurotrophic factors in differentiating ES cells. Factors were added to cultures from day 7 and the numbers of mDA neurons were determined at day14 by immunostaining with GFP and TH antibodies, respectively. As shown in Fig 6D, cultures treated with GDNF gave rise to 55% more *Pitx3*-GFP$^+$ neurons compared with medium controls. mDA neuron production was also enhanced by 34% and 22%, respectively, in cultures treated with NT3 and IGF1. However, no effect was observed for BDNF, TGFα, and FGF4 in the production of DA neurons under this culture paradigm. This data suggests that GDNF

is a potent factor in promoting differentiation and/or survival of ES-derived mDA neurons.

N-4-methyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is a toxin which selectively ablate dopaminergic neurons. To ask whether GDNF can protect *in vitro* generated mDA neurons from MPTP induced cell death, differentiating ES cells were cultured with GDNF as described above followed by treatment with 100 μM MPTP in the last two days of culture (day 12-14). The number of Pitx3-GFP$^+$ and TH$^+$ neurons were determined at day 14. MPTP treatment resulted in a 66% reduction of *Pitx3*-GFP$^+$TH$^+$ neurons compared to medium control cultures. However, in cultures pre-treated with GDNF this reduction was 30% (Fig 6E). This result indicates that, similar to primary mDA neurons, *in vitro* generated mDA neurons are sensitive to MPTP and that GDNF can protect ES-derived mDA neurons from MPTP mediated neuron toxicity.

Discussion

We have generated embryonic stem cells and a line of transgenic mice which express eGFP reporter under the control of endogenous *Pitx3* regulatory elements by gene targeting. Expression of *Pitx3*–GFP was visualised in the ventral midbrain where DA neurons reside, the ocular lens, head mesenchyme, somites, and muscles in the head region. This expression pattern recapitulates that of the *Pitx3* transcript during development (Semina et al., 2000a; Smidt et al., 1997).

In contrast to other transcription factor genes (*nurr 1, lmx1b*) which expression extends largely beyond the mDA neurons (Asbreuk et al., 2002; Saucedo-Cardenas et al., 1998;

Zetterstrom et al., 1996), Pitx3 has been shown to be restricted to DA neurons in the adult midbrain (Smidt et al., 1997; Van Den Munckhof et al., 2003). We show that, within the central nervous system, *Pitx3*–GFP expression was confined to TH-expressing neurons in the SN and VTA of the ventral midbrain, but not in DA neurons of other brain regions. Therefore the observed *Pitx3*–GFP expression agrees with reported expression pattern for *Pitx3* RNA (Smidt et al., 1997). However, we did not observe preferential distribution of *Pitx3*-GFP in the ventral aspect of SN DA neurons as described by van den Munckhof et al (Van Den Munckhof et al., 2003). It is possible that this apparent discrepancy is due to a difference in sensitivity of the read out system employed in the two studies. An alternative explanation might lie in a potential difference between Pitx3 RNA and protein expression. Since our GFP reporter was targeted upstream of the first coding exon (as opposed to an in-frame fusion downstream of translation start codon), *Pitx3*-GFP expression is more likely to mimic *Pitx3* transcripts.

DA neurons constitute less than 5% of mesencephalic cell preparation. Such low representation complicates cellular and molecular analysis of these pharmacologically and medically important cells. It may have also in the past accounted for the poor success in identifying mDA-specific genes. The highly restricted expression of vital GFP reporter in mDA neurons in the *Pitx3*-GFP mice provides, for the first time, a mean for direct isolation of late mDA progenitors and nascent mDA neurons by FACS sorting. We showed that this approach is feasible in *Pitx3* heterozygous embryos. Purified mDA neurons will allow detailed molecular analysis associated with DA phenotype and facilitate the identification of mDA regulator genes.

Neurotrophic factors, GDNF in particular promote differentiation, survival and maintenance of primary mDA neurons in cultures (Gash et al., 1998). Furthermore, *in vitro* and *in vivo* assays have shown that GDNF exerts strong protective effect on developing and adult mDA neurons (Beck, 1994; Eggert et al., 1999; Gill et al., 2003; Grondin and Gash, 1998). We have shown that, amongst several neurotrophic factors tested, GDNF was the most potent factor in promoting the production of mDA neurons from ES cells. In addition, GDNF protected *in vitro* generated DA neurons from MPTP mediated cell death. These results support the conclusion that, ES cell-derived DA neurons are not only similar in antigenic properties, but are also biologically similar to primary DA neurons in the embryonic and adult brain.

We have previously established protocols for efficient generation of neuroepithelial stem cells from ES cells by exploiting Sox-B expression (Li et al., 1998; Ying et al., 2003). Such system has been employed in screening for key molecules regulating neural differentiation by microarray and cDNA subtraction, as well as being used as a tool for functional testing of gene candidates derived from the screen (Aubert et al., 2002; Aubert et al., 2003). We anticipate that the *Pitx3*-GFP (PTG2) ES cell line and transgenic mice would provide a model system to track the fate of mDA neuron differentiation from ES cells and/or neural stem cells. Transplantation studies in animal models of Parkinson's disease demonstrated that synaptic formation between grafted DA neurons and striatal target cells is specifically associated with DA neurons of midbrain origin (Hudson et al., 1994). Therefore Pitx3 directed reporter expression would be a more physiologically

relevant monitor for mDA phenotype. In combination with transgene expression or knock down approaches in ES cells (Fujikura et al., 2002; Kim et al., 2002; Wagner et al., 1999), the *Pitx3*-GFP system could be used for identifying key determinants governing mDA fate and to investigate mechanisms of regulatory genes in mDA neuron differentiation. The *Pitx3*-GFP (PTG2) ES cells could also be used for pharmacological assays to analyse the activity of potential drugs and as a source of mDA neurons for transplantation studies in animal models of Parkinson's disease.

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Acknowledgments

We thank Jan Vrana for assistance in Flow Cytometry, Carol Manson and her staff for animal husbandry. M. L. is thankful to Austin Smith for his constant encouragement and support. This work is supported by grants from the UK Medical Research Council (MRC) and a MRC-Stem Cell Science UK Ltd Collaborative PhD studentship (S. M).

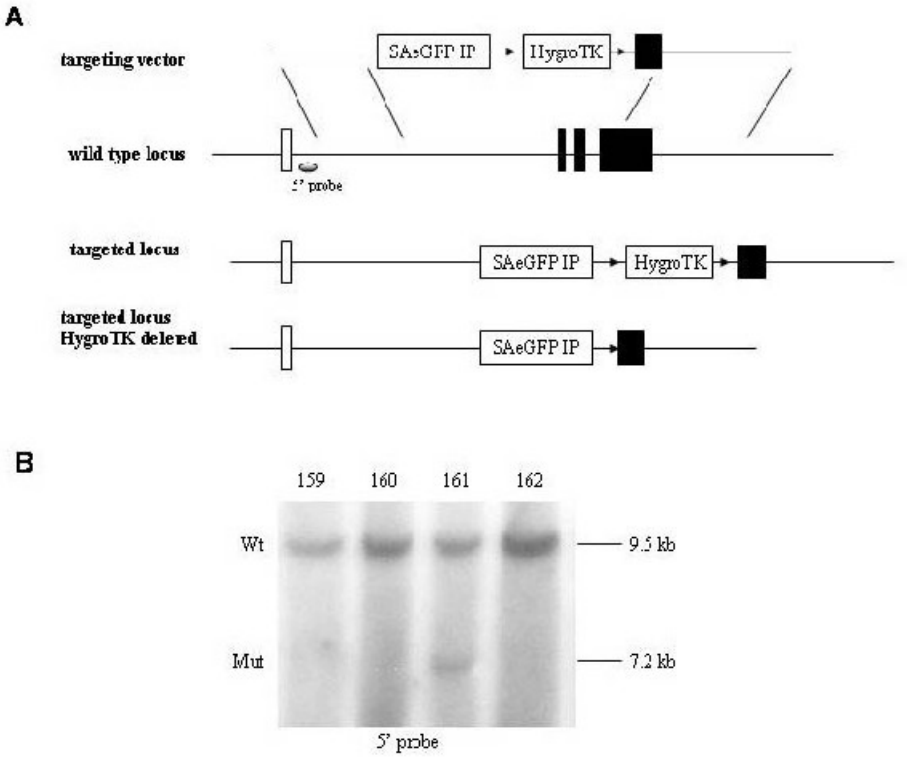

Fig 1. Targeting of the Pitx3 locus.

A, top to bottom: *Pitx3* KO vector, containing genomic *Pitx3* sequence for homologous recombination, a splice acceptor-egfp-ires-pac-pA (SAeGFPIP) cassette and a loxP – flanked cassette with a PGK-hygrotk positive/negative selectable marker; the wild type Pitx3 locus, with the location of the probe indicated; the structure of the targeted locus before and after Cre-mediated excision of the loxP-flanked PGK-hygrotk cassette. LoxP sites are indicated by solid arrowheads, the empty box denotes *Pitx3* exon 1 whilst the filled boxes represent exons 2-4.

B, Southern blot of DNA from 4 ES cell lines, digested with Hind III and hybridized with the probe indicated in A. The 9.5 kb band is the wild type band and the 7.2 kb band

represents the targeted allele. Line 159,160 and 162 are wild type, while line 161 is targeted.

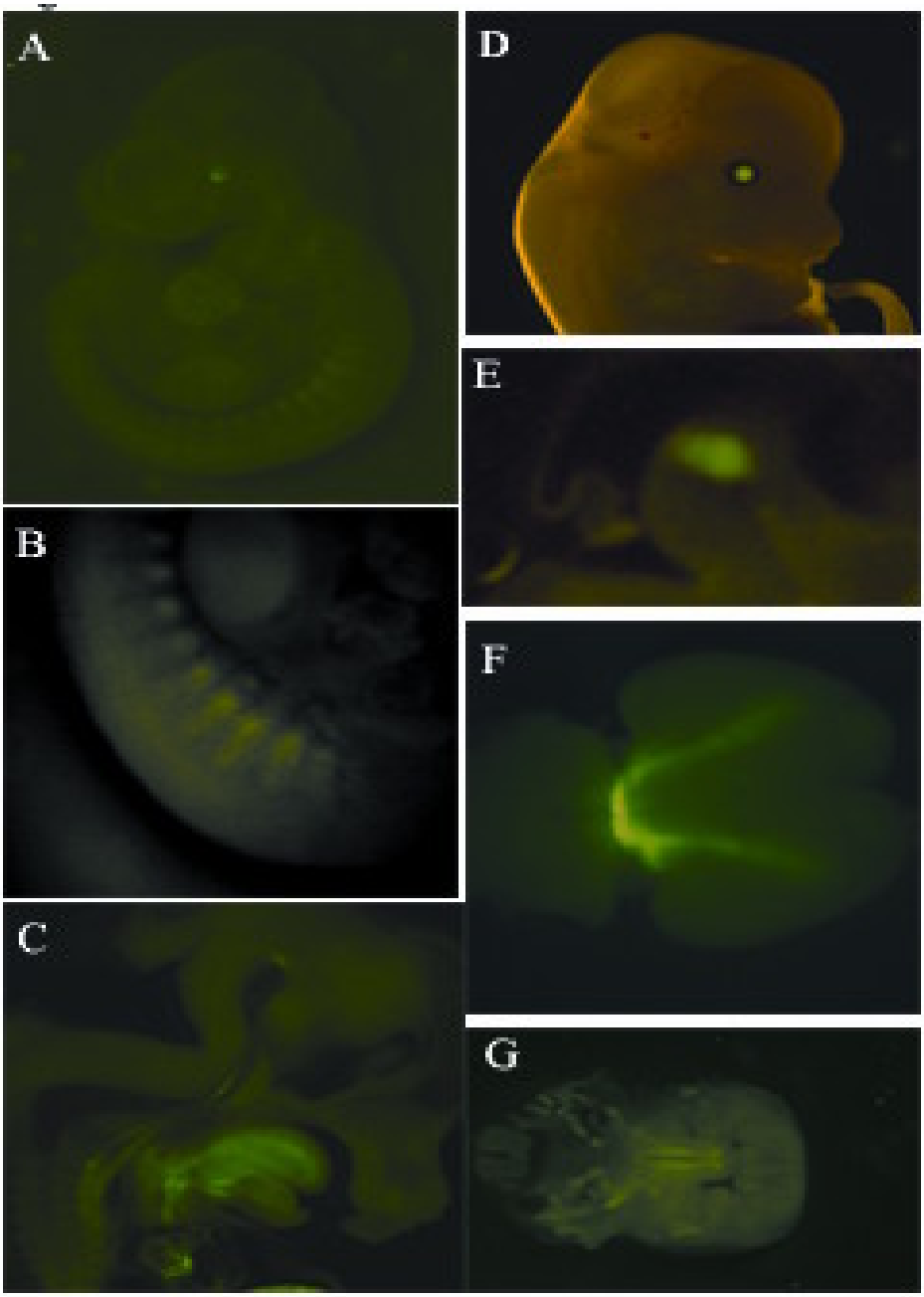

Fig 2. Direct visualization of *Pitx3*-GFP in developing mouse emrbyos. Transgenic mice were heterozygous for *Pitx3* locus.

A, GFP expression in the lens and somites in an E10 embryo.

B, a close up view of the same embryo at the trunk region showing somite expression.

C, a sagittal section of E12 embryo showing GFP expression in the ventral mesencephalon, muscle in tongue and cranial fiscal region.

D, GFP expression in the lens and mesenchyme of an E12.5 embryo.

E, a sagittal section of E13.5 embryo showing GFP expression in the ventral mesencephalon.

F, a ventral view of an E14.5 brain showing GFP expression in the midbrain and axonal projection to the forebrain.

G, a cross section of E14.5 head showing GFP expression in head muscles and surrounding mesenchyme.

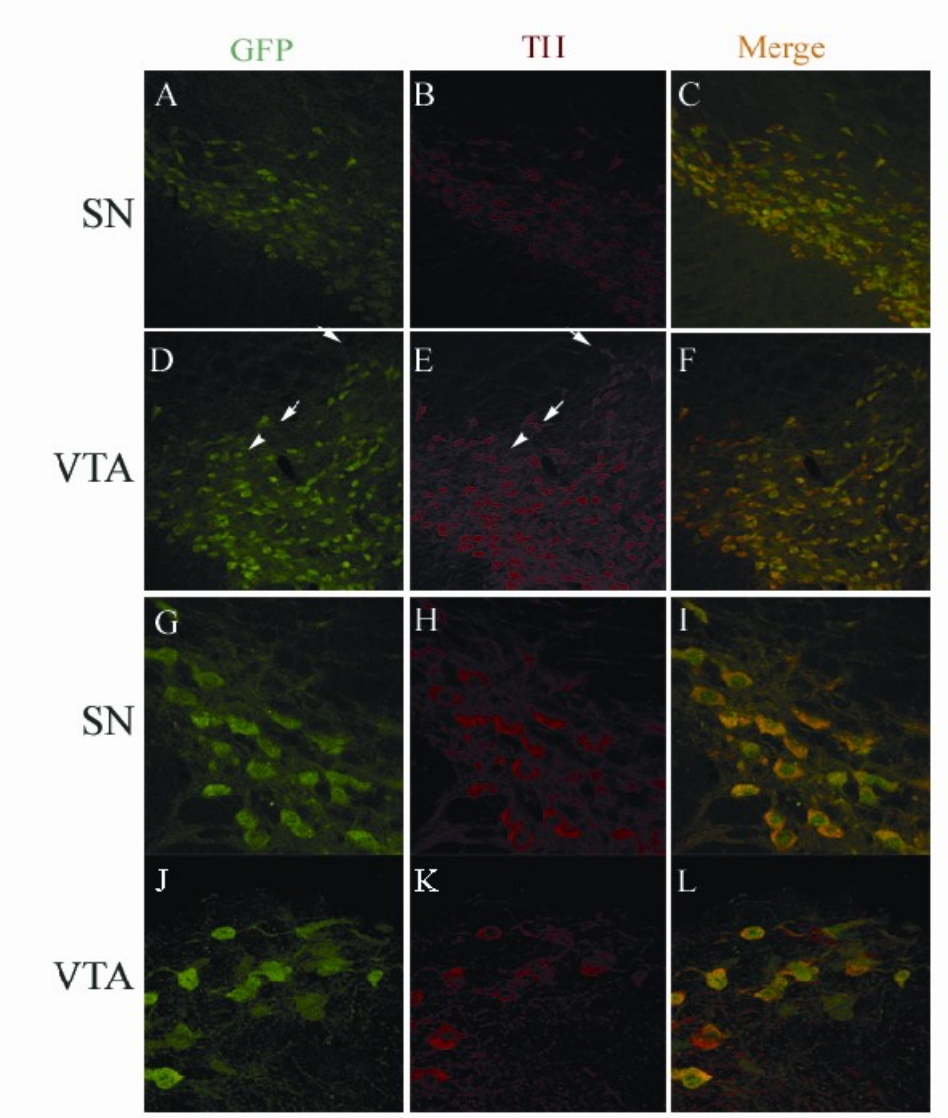

Fig 3. Co-localisation of *Pitx3*-GFP and TH in the adult ventral midbrain. A *Pitx3* heterozygous adult brain was sectioned on a frozen cryotome (30μm) and double labelled with GFP (green) and TH (red) antibodies. The vast majority of cells in the SN and VTA were positive for *Pitx3*-GFP and TH. Arrows in E point TH single positive cells whilst arrowheads in D indicate cells that were labelled with GFP only. A-F, 20x objective; G-L, 63x objective.

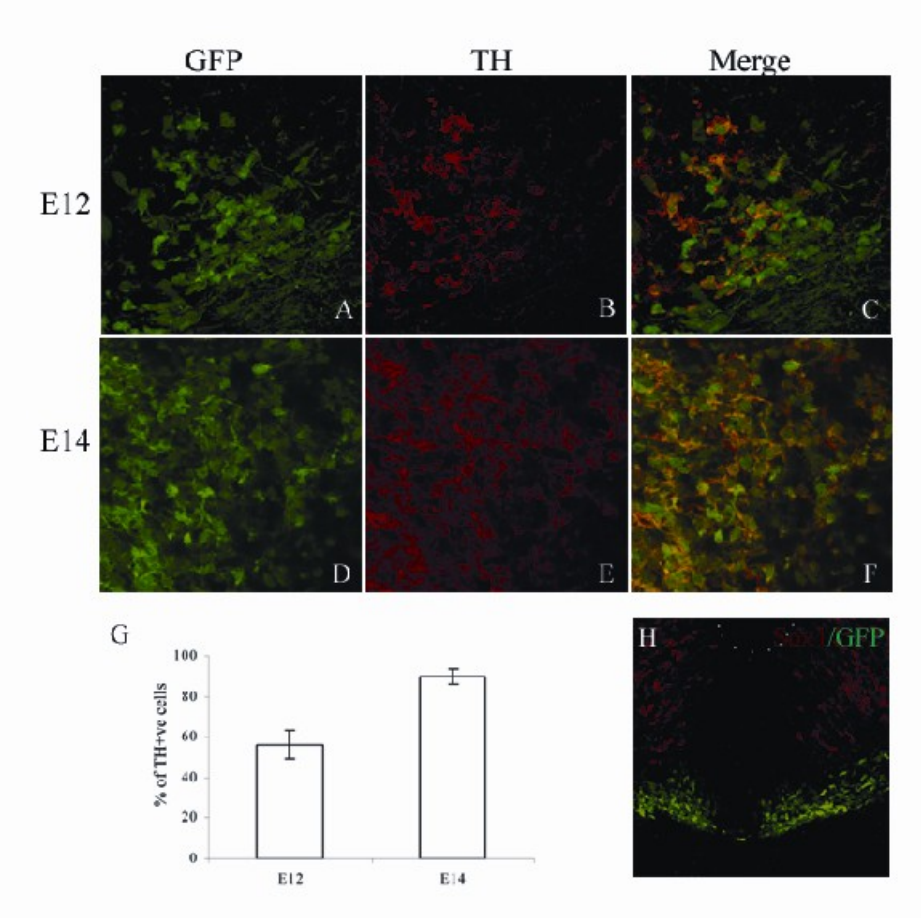

Fig 4. Pitx3-GFP expression in developing mesencephalon

Coronal sections of E12 and E14 heterozygous heads were double labelled with GFP (green) and TH (red) antibodies.

A-C, A section of an E12 head showing partial overlapping between GFP (A) and TH (B), C is an overlay of A and B.

D-F, An example of an E14 section. Note that most of the *Pitx3*-GFP^{+} cells were also TH^{+} at this stage.

G, Data shows the percentage of TH^{+} cells in the *Pitx3*- GFP^{+} population of E12 and E14 brain. Data was obtained from staining of 3 brains for each developmental stage.

H, Double antibody staining for Sox1 and GFP on an E12 midbrain section. *Pitx3*-GFP^{+} cells do not express mitotic neural progenitor marker Sox1. Dotted line indicates the edge of the ventral midbrain.

Fig5

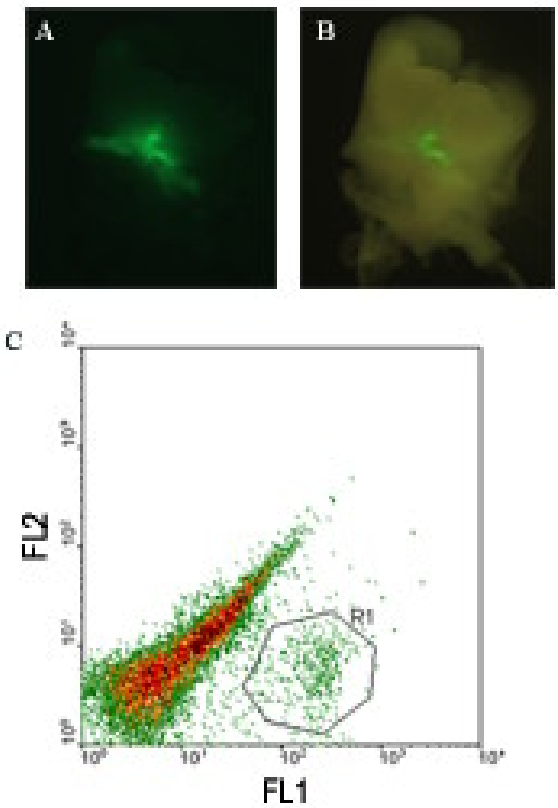

Fig 5. FACS sorting of mDA neurons from mouse embryos.

A-B, Shown is a dissected midbrain region to be prepared for flow sorting.

C, A FACS profile of E16 mesencephalic cell preparation.

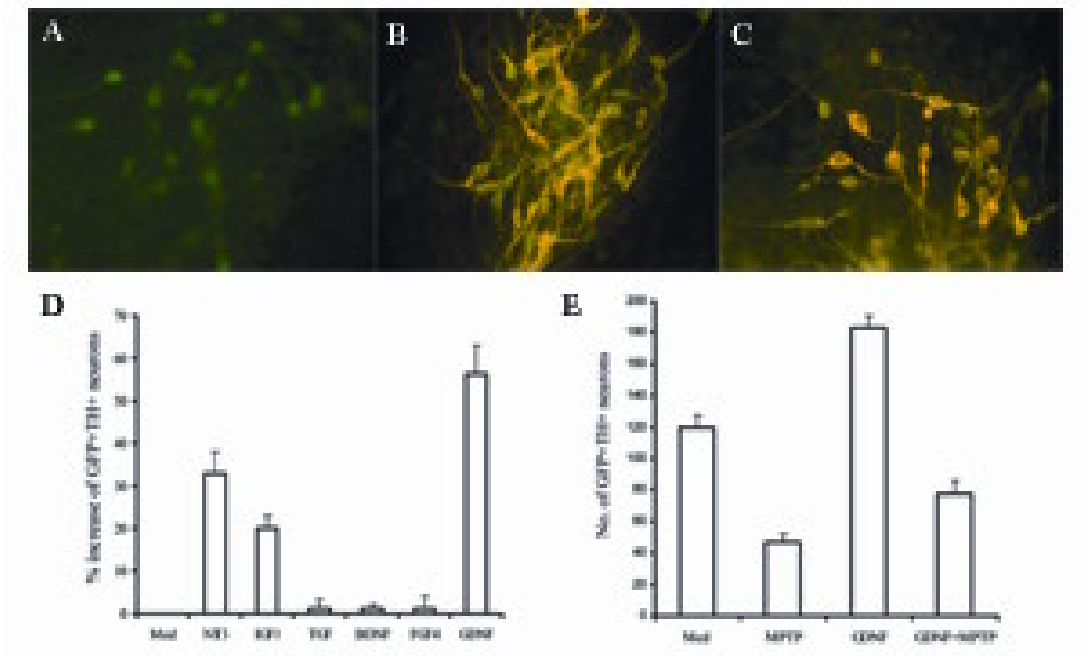

Fig 6 Analysis of ES cell-derived DA neurons suing the *Pitx3*-GFP reporter cells.

A, Direct visualisation of GFP in ES-derived neurons at day 12 of in vitro differentiation.

B-C, Double immunostaining of GFP (green) and TH (yellow) of a day14 differentiation culture.

D, GDNF promotes mDA neuron production from ES cells. PTG2 ES cells were co-cultured with PA6 for 7 days in serum free medium followed by another 7 days with added neurotrophic factors. The numbers of GFP^+ and TH^+ cells were determined by double antibody staining.

E, GDNF protects ES-derived DA neurons from MPTP-induced cell death. GDNF was added from day 10 followed by MPTP treatment at day 12. Cultures were terminated at day 14 and the numbers of GFP expressing cells were determined.

Table 1. *Pitx3*-GFP co-localise with TH in mDA neurons

	Number of cells counted	*Pitx3*-GFP$^+$% TH$^+$	TH$^+$% *Pitx3*-GFP$^+$
SN	884	98.35±2.3	99.36±0.9
VTA	457	94.6±8.5	94.9±6.4

Counting was performed on sequentially scanned confocal image series taken with 20x or 63x objectives. Data was collected from eight randomly chosen fields of three adult brain samples.

Table 2, Purification of primary mDA neurons by flow cytometry.

Age of embryos (number of embryos)	% GFP$^+$ cells (mean ± SD)	No. of GFP$^+$ cells per midbrain (mean ± SD)
E12 (n=20)[a]	0.6 ± 0.4	537 ± 271
E13 (n=12)[b]	1.3 ± 1.1	801 ± 227
E16 (n=2)[c]	3.89	1500

a, data obtained from 3 independent FACS sorting of a pool of 9, 5 and 6 midbrains, respectively.

b, collective data of 5 separate sorting experiments of a total of 12 midbrains.

c, A single sorting of cell preparation made from 2 midbrains.

Table 3, DA neuron marker expression by ES-derived *Pitx3+* cells.

Additional treatment	Neuronal markers	Marker$^+$	Marker$^+$GFP$^+$	GFP$^+$ only
Non	TH	1076±89	146±7	14.2±2.1
	DAT	346±42	54±9	107±12.5
	βTubulin3	2543±301	173±26	4.4±3.7
Shh & FGF8	TH	3128±421	635.7±87	106±12.1

Day 14 cultures were fixed with 4% PFA and processed for antibody staining using antibodies against GFP and the markers indicated.